EXHIBIT A

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Cationic Liposomes Containing Mycobacterial Lipids: a New Powerful Th1 Adjuvant System

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The immunostimulation provided by the mycobacterial cell wall has been exploited for many decades, e.g., in Freund's complete adjuvant. Recently, the underlying mechanism bebind this adjuvant activity, including Toll receptor signaling, bas begun to be unraveled, confirming the potential of mycobacterial constituents to act as adjuvants. In this study, the immunostimulatory properties of a *Mycobacterium bovis* BCG lipid extract were tested for their adjuvant activity. Administration of the lipids in dimethyl dioctadecyl ammonium bromide-based cationic liposomes induced a powerful Th1 response characterized by markedly elevated antigen-specific immunoglobulin G2a (IgG2a) isotype antibodies and substantial production of gamma interferon. The adjuvant formulation (designated mycosomes) elicited high levels of gamma interferon both in C57BL/6 as well as in Th2-prone BALB/c mice. Furthermore, the mycosomes induced immune responses to protein antigens from several sources including *Mycobacterium tuberculosis*, *Chlamydia muridarum*, and tetanus toxoid. In a tuberculosis challenge model, the mycosomes combined with the Ag85B-ESAT-6 fusion protein were demonstrated to have a unique ability to maintain sustained immunological memory at a level superior to live BCG.

Vaccine research in recent years, both in the infectious disease and cancer fields, has highlighted the need for effective adjuvant formulations that induce cellular immune responses. The need for adequate adjuvants applies not only to vaccines based on recombinant proteins and synthetic peptides of inherently low immunogenicity but also to coadministration with other constructs such as DNA vaccines and adenoviral vectors (29, 42). Despite the general need for robust adjuvants and delivery systems for modern vaccines, the only adjuvants approved for human use worldwide are, even today, Th2-promoting aluminum-based compounds. This has led to a major interest in the development of Th1-inducing adjuvants for human use. Although a number of candidates have been evaluated (32), a Th1 adjuvant for human use has yet to be approved.

Improved understanding of the initiation of immune responses and, in particular, the discovery of receptors recognizing microbial constituents, has revealed a new strategy for adjuvant research. By mimicking these pathogen-associated molecular patterns, it is now possible to design synthetic analogues that act as ligands for these receptors. In this context, a panel of synthetic lipid A derivatives has been designed to serve as agonists for human Toll-like receptor 4 (TLR4) (6), while other well-known adjuvants, such as unmethylated CpG DNA motifs, have been found to be ligands for TLR9. However, given the complexity of the events leading to a protective immune response, the new generation of adjuvants is unlikely to be based on a single component. Rather, more complex adjuvant formulations based on combinations of several monotherapeutic agents capable of targeting multiple different receptors, and therefore more likely to induce complex and sufficient immune responses, are seen as the way forward (31). One example of this development is represented by the development of the archaeosomes based on the polar lipid fraction from archaea. The archaeosome adjuvant system has been characterized in detail by Sprott and coworkers, and it is able to induce a humoral as well as a cell-mediated immune response (25).

Preparations of mycobacteria have been shown to exert their effect by signaling through several TLRs (8) and have long been recognized as an important source of immunostimulants. We have investigated the immunostimulatory capacity of a total lipid extract of *Mycobacterium bovis* BCG obtained using a simple purification process suited for large-scale production. The lipid extract, delivered in cationic liposomes based on dimethyl dioctadecyl ammonium bromide (DDA) and designated mycosomes, was found to generate strong antigen-specific immune responses to a range of different antigens. This immune response was characterized by high levels of antibodies and gamma interferon (IFN-γ) and a very efficient maintenance of immunological memory.

MATERIALS AND METHODS

Reagents. DDA was obtained from Eastman Kodak, Inc. (Rochester, NY). Phosphatidylglycerol (PG), L-phosphatidylcholine (PC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium chloride (DOTAP), and cholesteryl 3b-*N*-(dimethylaminoethyl)carbamate hydrochloride (DC-Chol) were all purchased from Sigma Aldrich Denmark (Brøndby, Denmark). Alum (2% alhydrogel) was from Brentag Biosector (Frederikssund, Denmark), and lipid A was from Avanti Polar Lipids (Alabama).

Extraction of lipids. *M. bovis* BCG was cultured in modified Sauton medium (2). The mycobacteria were harvested after 2 to 3 weeks of culturing, suspended

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TABLE 1. Overview of the subunit vaccines used for immunization of mice

Antigen	Adjuvant
	100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	250 μg of DDA
2 μg of Ag85B-ESAT-6	100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	None
10 μg of MOMP	None
10 μg of ovalbumin	None
10 μg of tetanus toxoid	None
2 μg of Ag85B-ESAT-6	Mycosomes (250 μg of DDA/100 μg of BCG lipids)
10 μg of MOMP	Mycosomes (250 μg of DDA/100 μg of BCG lipids)
10 μg of ovalbumin	Mycosomes (250 μg of DDA/100 μg of BCG lipids)
10 μg of tetanus toxoid	Mycosomes (250 μg of DDA/100 μg of BCG lipids)
2 μg of Ag85B-ESAT-6	250 μg of DDA/25 μg of MPL
2 μg of Ag85B-ESAT-6	250 μg of DOTAP/100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	250 μg of DC-Chol/100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	250 μg of DOPE/PC/100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	250 μg of DOPE/PC/PG/100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	500 μg of alum

in phosphate-buffered saline (PBS), and incubated for 1.5 h hours at 60°C. After centrifugation and removal of the supernatant, lipids were extracted by treating 10 to 15 g of mycobacteria (wet weight) with 30 ml of chloroform-methanol (2:1) for 15 min at 55°C. The extraction was repeated, and the organic phases from both extractions were pooled and washed twice with 5 ml of water to remove hydrophilic molecules. The solvent of the organic phases was evaporated, and the amount of dry lipid material was weighed, redissolved in chloroform, and aliquoted into vials of 1 or 5 mg, followed by evaporation of chloroform and storage at −20°C.

Antigens. The fusion protein of Ag85B and ESAT-6 (hereafter designated Ag85B-ESAT-6) was produced as a recombinant protein as previously described (33). Ovalbumin was obtained from Sigma; tetanus toxoid was from Statens Serum Institut, Copenhagen, Denmark. The recombinant major outer membrane protein (MOMP) from *Chlamydia muridarum* was expressed in the pDest17 system (Gateway; Invitrogen) and purified as previously described (40).

Adjuvants and vaccines. Total lipid extracts were prepared by rehydrating dry *M. bovis* BCG lipid material with Milli Q water at 1 or 5 mg/ml, followed by probe sonication on a Sanyo Soniprep 150 MSE sonicator (2 pulses of 30 s at amplitude of 10 μm).

DDA was prepared by adding DDA powder to sterile distilled water (2.5 mg/ml) and heating at 80°C under continuous stirring for 20 min, followed by cooling to room temperature before use. The standard mycosome vaccine was prepared by mixing the antigen with saline, followed by the addition of rehydrated lipid extract and DDA and vortex mixing. The vaccine was left overnight to allow adsorption of the antigen.

Other liposomes were composed of DOTAP, DC-Chol, PC-DOPE (neutral liposomes; molar ratio of 1:0.5) or PC-DOPE-PG (anionic liposomes; molar ratio of 1:0.5:0.25). Vaccines for a total of five mice were prepared by evaporation of solvent from 1.25 g of the total liposome-forming compound(s) dissolved in chloroform. The dry lipid material was hydrated with 500 μl of Milli Q water and sonicated for 30 min in a bath-type sonicator. Ten micrograms of antigen in 100 μl of 50 mM ammoniumcarbonate buffer and 500 μl of BCG lipids (1 mg/ml) were added, followed by lyophilization. The lipid-antigen mixture was rehydrated by the addition of 1,000 μl of saline.

Alum was added to the antigen mixed with saline immediately before immunization. DDA-monophosphoryl lipid A (MPL) was prepared as previously described (10). An overview of the various adjuvant preparations used in this study is provided in Table 1.

Characterization of BCG total lipid extract. Two-dimensional thin layer chromatography (2D-TLC) of *M. bovis* BCG lipid extracts was performed by Claire Reid at the Scottish Crop Research Institute according to the method of Dobson et al. (13). A total of 1.4 mg of lipid material dissolved in chloroform-methanol (2:1) was applied for each analysis. An *M. tuberculosis* standard lipid extract prepared and characterized as previously described (13) was used as a standard for the TLC.

Apolar lipids were analyzed in the following system: first direction, petroleum ether (bp 40 to 60°C)-ethyl acetate (98:2); second direction, petroleum ether (bp 40 to 60°C)-acetone (98:2). Nonpolar lipids were detected with 20% molybdophosphoric acid in ethanol and heated at 120°C.

Polar lipids were analyzed in the following system: first direction, chloroform-methanol-water (60:30:6); second direction, chloroform-acetone-methanol-water (47:25:3:5). Polar lipids were detected with 20% molybdophosphoric acid in ethanol and heated at 120°C; ninhydrin reagent was used to detect lipids with free amino groups, and Phospray (Sigma) was used to detect phospholipids.

Glycolipids of intermediate polarity were analyzed in the following system: first direction, chloroform-metbanol-water (100:14:0.8); second direction, chloroform-acetone-methanol-water (50:60:2.5:3). Glycolipids were detected by α-naphthol reagent and heating at 110°C.

Ten-microliter samples of rehydrated lipid extracts (1 mg/ml) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27) and silver staining (9) for residual protein content.

The adsorption of antigen to mycosomes was investigated by mixing 50 μg of antigen and 0.5 mg of rehydrated lipid extract prepared as described above with 1.25 mg of DDA in a total volume of 1 ml. Adsorption was allowed to proceed overnight. The samples were then ultracentrifuged (100,000 × *g* for 1 h). The supernatant was collected and the pellet was resuspended in the original volume (1 ml); both samples were analyzed by SDS-PAGE and silver staining. Protein concentrations in the supernatants were determined by the microbicinchoninic acid method according to the manufacturer's instructions (Pierce Europe, Oud-Beijerland, The Netherlands), and the amount of antigen adsorbed to the mycosomes was determined as previously described for DDA-BBG2Na solutions (23).

Particle size analysis was performed by photon correlation spectroscopy using a Malvern Zetasizer 4 with a ZET 5110 cell (Malvern Instruments, Ltd., Worcestershire, United Kingdom). The *Z*-average diameter and the polydispersity index (PI) were determined. Small values of PI (<0.1) indicate a population of low heterogeneity, while PI values of >0.3 indicate high heterogeneity.

A test for pyrogenicity in the standard rabbit model was performed by Charles River (Wiga, Germany) according to the European Pharmacopoeia. Rabbits were given an intravenous dose (0.5 ml/kg of body weight) of different concentrations of mycosomes, and rectal temperatures were recorded for 3 h after administration.

Animals. Female BALB/c or C57BL/6 mice, 8 to 12 weeks old, were obtained from Bomholtgaard (Ry, Denmark) or Harlan Scandinavia (Denmark). Infected mice were kept in cages within a BL-3 laminar flow safety enclosure.

Immunization. Mice were immunized subcutaneously (s.c.) with vaccines containing 0.5 μg (adjuvant controls), 2 μg (Ag85B-ESAT-6), or 10 μg of the antigen (all other antigens) in a total volume of 0.2 ml, at the base of the tails three times with a 2-week interval between each immunization. As a positive control in the experiment involving *M. tuberculosis* infection, a single group of mice received one dose of BCG Danish 1331, 5×10^6 CFU, injected s.c. at the base of the tail.

Determination of antibody titers. Plates for enzyme-linked immunosorbent assay (Nunc maxisorp, Roskilde, Denmark) were coated with ovalbumin (2 μg/well), Ag85B-ESAT-6, MOMP, or tetanus toxoid (0.05 μg/well) in PBS overnight at 4°C. Free binding sites were blocked with PBS containing 2% skim milk. Individual mouse serum from three to four mice per group was analyzed in duplicate in fivefold dilutions at least 10 times in PBS with 1% bovine serum albumin; the initial dilution was 20-fold. After a washing procedure, horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit anti-mouse immunoglobulin G1 [IgG1] and IgG2a; Zymed) diluted 1/2000 in PBS containing 1% bovine serum albumin was added. Following 1 h of incubation, antigen-specific antibodies were detected by TMB (3,3′,5,5′-tetramethylbenzidine) substrate as described by the manufacturer (Kem-En-Tec, Copenhagen, Denmark). The absorbance values were plotted as a function of the reciprocal dilution of serum samples. The data were fitted by nonlinear regression analysis with a sigmoidal dose-response curve of variable slope by the GraphPad Prism program (version 4.00; GraphPad Software Inc.). Antibody titers were then defined as the serum dilution that gives an absorbance value of 1.0 in the parallel portion of the curves (39). For serum samples where the antibody titration curves were below this value, the titer was defined as below the dilution 20 (<20).

Cellular assays. Blood samples were drawn from mice 7 days after the last immunization, pooled from five to six mice in each group, and the blood lymphocytes were obtained (3). Splenocytes were isolated from mice 7 days after the last immunization as previously described (3). Cell cultures were performed in

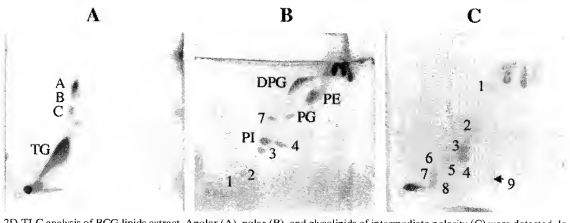

FIG. 1. 2D-TLC analysis of BCG lipids extract. Apolar (A), polar (B), and glycolipids of intermediate polarity (C) were detected. In the apolar fraction (panel A), triacylglycerol (TG) and phthiocerol dimycocerosate A, B, and C (A, B, and C) were detected. The polar fraction in panel B contained phosphatidylinositol mannosides (1–4), phosphatidylinositol (PI), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), and L-alpha-phosphatidyl-DL-glycerol (PG). In the fraction of intermediate polarity shown in panel C, the numbers 1 to 9 indicate glycolipids of intermediate polarity that were not identified.

triplicate in round-bottomed microtiter wells containing 2×10^5 cells in a volume of 200 μl RPMI medium supplemented with 2-mercaptoethanol, glutamine, penicillin-streptomycin, HEPES, and 10% fetal calf serum. Antigens were used in concentrations ranging from 5 to 0.08 μg/ml. Wells containing medium only or 5 μg/ml of concanavalin A were included in all experiments as negative and positive controls, respectively. Culture supernatants were harvested from parallel cultures after 72 h of incubation in the presence of antigen, and the amount of IFN-γ was determined by enzyme-linked immunosorbent assay using purified rat anti-mouse IFN-γ (BD Pharmingen, San Diego, CA) as the coating antibody, biotin-labeled rat anti-mouse IFN-γ (BD Pharmingen) as capture antibody, and HRP-conjugated streptavidin (Zymed, San Francisco, CA) for detection of IFN-γ. The presence of interleukin-5 (IL-5) was analyzed similarly using anti-mouse IL-5 coating and capture antibodies (BD Pharmingen) and HRP-conjugated streptavidin (Zymed).

To evaluate the responding T-cell subset, the CD4 and CD8 T-cell receptors were blocked as previously described (1).

Fluorescence-activated cell sorting analysis. Splenocytes were isolated from mice 7 days after the last immunization and restimulated in 96-well U-bottom plates containing 5 μg/ml of Ag85B-ESAT-6 and 2×10^6 cells/well. Control wells without antigen were also included. After restimulation overnight, brefeldin A (Sigma) was added to a final concentration of 2.25 μg/well, and the cultures were further incubated for 4 h. After cells were washed, nonspecific binding was blocked by a 15-min incubation with the 24G2 clone (CD16/CD32; BD Pharmingen) and subsequently stained with peridinin chlorophyll protein-CD4 and allophycocyanin-CD8 (both BD Pharmingen) on ice for 20 min. Intracellular cytokine staining was performed using the Cytofix/Cytoperm kit available from BD Pharmingen according to the manufacturer's protocol and using phycoerythrin–IFN-γ (BD Pharmingen). Cells were finally washed three times, resuspended in paraformaldehyde, and analyzed with a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA) by collecting 50,000 events.

Experimental infections. For evaluation of vaccine efficacy, mice were challenged 2.5, 6, or 14 months after the first immunization by the aerosol route in a Glas-Col inhalation exposure system (Inhalation Exposure System 099C A4224; Glas-Col, IN) calibrated to deposit approximately 25 CFU of virulent *M. tuberculosis* Erdman in the lungs. The bacterial loads in spleen and lungs were determined 6 weeks later by plating serial dilutions onto Middlebrook 7H11 agar supplemented with 2 μl of 2-thiphene-carboxylic acid hydrazide per ml to selectively inhibit the growth of BCG. Colonies were counted after 2 to 3 weeks of incubation at 37°C.

Statistical analyses. Differences in numbers of colonies between infected mice and control mice were tested by analysis of variance. When significant effects were indicated, differences between means were assessed by a Dunnetts test.

RESULTS

Preparation and characterization of a mycobacterial total lipid extract. *M. bovis* BCG was chosen as the starting material for the preparation of a mycobacterial lipid extract obtained using a standard chloroform-methanol extraction method. The total lipid composition of the extract was analyzed by 2D-TLC for apolar and polar lipids and lipids of intermediate polarity, according to the method outlined by Dobson et al. (13). Of the apolar lipids present, phthiocerol dimycocerosates A, B, and C and triacylglycerol were identified (Fig. 1A). The polar lipids identified were phosphatidylinositol mannosides, phosphatidylinositol, phosphatidylethanolamine, and diphosphatidylglycerol (Fig. 1B). Small amounts of L-alpha-phosphatidyl-DL-glycerol sodium salt and an unknown phospholipid were also detected. Nine glycolipids of intermediate polarity were detected in the lipid extract. These lipids were not identified (Fig. 1C). Comparison of the 2D-TLCs obtained for three independent extractions of BCG lipids showed the same overall TLC profiles (data not shown).

The lipid extract was subsequently tested for protein contamination by silver-stained SDS-PAGE. The presence of protein bands was not detected even after extensive development of the gel (data not shown). Before the lipid extract was used for immunization, the pyrogenicity of the lipids was tested in an established rabbit pyrogenicity model. The results from this model demonstrated that the lipids were not pyrogenic even at concentrations up to 1.0 mg/ml (results not shown).

Liposomes as vehicles for mycobacterial lipids. A range of different cationic, neutral, and anionic lipid formulations have previously been used as vehicles for immunomodulators to obtain efficient adjuvant systems. Therefore, the ability of BCG lipids to modulate immune responses when delivered entrapped in liposomes of different charges was investigated. In this study, the tuberculosis (TB) subunit candidate, Ag85B-ESAT-6, was used as a model antigen. Ag85B-ESAT-6 was administered in combination with the BCG lipids in cationic liposomes formed of either DDA, DOTAP, or DC-Chol; neutral liposomes formed by DOPE-PC; and anionic liposomes formed by DOPE-PC-PG. For comparison, Ag85B-ESAT-6 was also administered in the traditional adjuvant, alum. Immune responses were monitored by in vitro restimulation of peripheral blood mononuclear cells purified 1 week after the

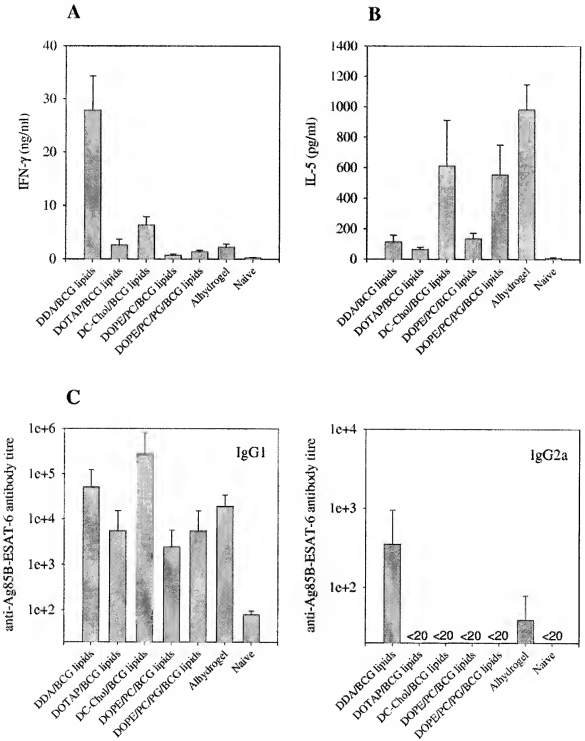

FIG. 2. Immune responses generated by BCG lipids entrapped in liposomes of different charges. Release of IFN-γ (A) or IL-5 (B) from blood lymphocytes isolated from BALB/c mice immunized with 2 μg of Ag85B-ESAT-6 in DDA-BCG lipids, DOTAP-BCG lipids, DC-Chol-BCG lipids, DOPE-PC-BCG lipids, DOPE-PC-PG-BCG lipids, or naive mice. Blood lymphocytes were isolated 5 weeks after the first immunization and restimulated in vitro with Ag85B-ESAT-6 (5 μg/ml). (C) Antigen-specific antibody midpoint titers in serum from BALB/c mice immunized with Ag85B-ESAT-6 measured as IgG1 and IgG2a titers.

last immunization. As shown in Fig. 2A, BCG lipids together with DDA elicited the most pronounced levels of IFN-γ release. In contrast, IL-5 production was mainly seen in mice immunized with alum, DC-Chol, or DOPE-PC-PG, while DDA-BCG lipids only gave minimal levels of IL-5 (Fig. 2B). Analysis of antigen-specific antibodies demonstrated an efficient induction of both IgG1 and IgG2a antibodies by the combination of DDA-BCG lipids (Fig. 2C). The IgG1 titer

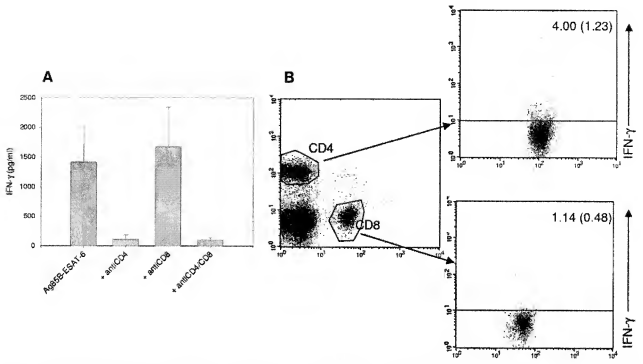

FIG. 3. Evaluation of the IFN-γ responses of CD4 and CD8 T-cell subsets. (A) Release of IFN-γ from spleen lymphocytes isolated from BALB/c mice immunized with 2 μg of Ag85B-ESAT-6 in mycosomes. Splenocytes were isolated 1 week after the first immunization and blocked with anti-CD4, anti-CD8, or anti-CD4/CD8 prior to restimulation with 5 μg/ml of Ag85B-ESAT-6. (B) Percentage of the CD4 and CD8 T-cell population positive for IFN-γ in the spleen of immunized mice. The percentage of IFN-γ positive cells in the spleens of nonimmunized control mice are indicated in the parentheses. The splenocytes were restimulated with Ag85B-ESAT-6 overnight. The graph is representative of three individual mice.

obtained with this combination was, in fact, 2.6-fold higher than when the antigen was administered in alum. Furthermore, although the IgG1 antibody titers were higher than the IgG2a titers, the ratio of IgG2a:IgG1 was clearly increased after administration of DDA-BCG lipids compared to both alum and the other combinations of liposomes and BCG lipids. Together, these results demonstrate the induction of an efficient Th1 cell-mediated immune response with the DDA-BCG lipids, and this combination (in the following referred to as mycosomes) was therefore subjected to further investigation. The immunological response was compared for three independent batches of BCG lipids, and the same level of IFN-γ release was obtained for the extracts (results not shown). In order to optimize the mycosomes, different amounts of lipids were tested together with the standard dose of 250 μg of DDA per mouse. These studies demonstrated an optimum dose of 100 μg of BCG lipids (results not shown).

For evaluation of the T-cell subset mediating the response observed with mycosomes, spleen cultures from immunized mice were blocked with monoclonal antibodies against the CD4 and CD8 receptors prior to restimulation with Ag85B-ESAT-6. As shown in Fig. 3A, the response was completely abrogated by anti-CD4, whereas no effect was observed by blocking CD8 T cells. Flow cytometry analysis of T cells and intracellular IFN-γ staining provided further evidence that the CD4 T cells are the responding subset in mycosome-immunized mice (Fig. 3B).

Mycosomes induce Th1 responses in C57BL/6 and BALB/c mice. DDA has previously been used in combination with immunomodulators such as MPL for enhancing their inherent adjuvant activity (10). For comparison, DDA-MPL as well as mycosomes were used as adjuvant formulations in two mice strains of different genetic backgrounds (11). Groups of C57BL/6 and BALB/c mice were immunized three times at 14-day intervals with 2 μg of Ag85B-ESAT-6, and the immune responses induced were assessed 5 weeks after the first vaccination. In C57BL/6 mice, both adjuvant combinations induced strong IFN-γ responses after restimulation with the vaccine antigen (Table 2). In BALB/c mice, in contrast, only the mycosomes induced a strong Th1 recall response to the Ag85B-ESAT-6 antigen with high levels of IFN-γ. DDA-MPL, in comparison, induced much more modest levels of IFN-γ in this Th2-biased mouse strain. The lipid extract exhibited no activity alone, emphasizing the necessity for a vehicle to maximize the immunostimulatory effects of the lipids.

Characterization of the mycosomes. Particle size analysis of DDA alone gave a *Z*-average size of 853 nm and a polydispersity index of 0.198, while the rehydrated BCG lipids showed a *Z*-average diameter of 290 nm and a polydispersity index of 0.553. The mycosomes gave a *Z*-average diameter of 738 nm and a polydispersity index of 0.300, indicating that a more homogenous particle size distribution is obtained compared to the BCG lipids on their own.

The amount of either Ag85B-ESAT-6 or ovalbumin ad-

TABLE 2. Ag85B-ESAT-6 responses in mice vaccinated with different adjuvant combinations

Vaccine[a]	IFN-γ (ng/ml) ± SD[b]	
	C57BL/6	BALB/c
Expt 1		
DDA	6.27 ± 2.93	ND
DDA/MPL	23.6 ± 7.50	0.40 ± 0.05
Mycosomes	118.2 ± 23.8	11.0 ± 0.10
Expt 2		
DDA	1.60 ± 0.40	ND
BCG lipids	0.06 ± 0.04	ND
Mycosomes	19.29 ± 0.29	ND

[a] Mice were immunized three times with 2 μg of Ag85B-ESAT-6 in the indicated adjuvant ($n = 3$ to 6 mice).

[b] Peripheral blood mononuclear cells were purified from the blood one week post the last immunization and restimulated with 5 μg/ml of Ag85B-ESAT-6. The presence of IFN-γ was measured in the supernatants using ELISA. ND not done.

sorbed to the mycosomes was analyzed by SDS-PAGE and silver staining after ultracentrifugation. The majority of the antigen was found in the adjuvant pellet of the vaccine, indicating a very efficient adsorption of both antigens to the mycosomes (Fig. 4). As a control, ultracentrifugation of the antigen solution alone demonstrated minimal precipitation or aggregation as the vast majority of the antigen was found in the supernatant (data not shown). Quantitation of the amount of protein in the supernatant by the microbicinchoninic protein assay showed that 89 and 88% of ovalbumin and Ag85B-ESAT-6, respectively, were found to be adsorbed to the mycosomes.

Mycosomes as adjuvants for different antigens. In this study, Ag85B-ESAT-6 was used in all the initial studies of immune responses promoted by mycosomes; we continued by comparing the adjuvant activity for a panel of nonmycobacterial antigens, namely, the MOMPs (major outer membrane proteins) from *C. muridarum*, tetanus toxoid, and ovalbumin. All antigens were administered together with our standard dose of mycosomes three times by the s.c. route, and immune responses were monitored 5 weeks after the first immunization.

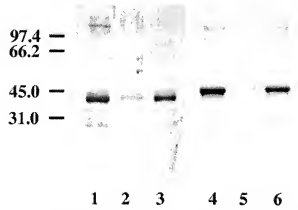

FIG. 4. Silver-stained SDS-PAGE gel of antigen adsorption to mycosomes. Lane 1, Ag85B-ESAT-6; lane 2, Ag85B-ESAT-6 in mycosome supernatant; lane 3, Ag85B-ESAT-6 in mycosome pellet; lane 4, ovalbumin; lane 5, ovalbumin in mycosome supernatant; lane 6, ovalbumin in mycosome pellet.

High levels of IFN-γ were recorded after immunization with MOMP and tetanus toxoid, whereas administration of ovalbumin in mycosomes gave rise to only modest levels of IFN-γ (Fig. 5). Both ovalbumin and the rest of the panel gave rise to high levels of IgG1 antibodies after immunization with the individual antigens. The IgG2a titers to each antigen were lower than the IgG1 titers but in agreement with the high IFN-γ levels; MOMP gave rise to the highest increase of IgG2a. Compared to administration of the antigens without adjuvant, the observed increase in IgG1 titers (n-fold) for MOMP, ovalbumin, and tetanus toxoid were 4.2, 970, and 47, respectively. For IgG2a, the observed increase in titers (n-fold) for MOMP, ovalbumin, and tetanus toxoid were 16, 4.2, and 5.2, respectively. These findings demonstrate that the mycosomes can be used to enhance immune responses to proteins with different characteristics and from sources other than *M. tuberculosis*.

Protective efficacy of the Ag85B-ESAT-6 delivered in mycosomes. In order to evaluate the actual efficacy of the Ag85B-ESAT-6 delivered in mycosomes against an *M. tuberculosis* challenge infection, BALB/c mice were immunized three times with Ag85B-ESAT-6 emulsified in mycosomes as well as the single components and challenged with virulent *M. tuberculosis* Erdman by the aerosol route 10 weeks after the first immunization. As shown in Table 3, Ag85B-ESAT-6 emulsified in mycosomes induced high levels of protection in both spleen and lungs, whereas a more modest efficacy was obtained using BCG lipids alone to emulsify Ag85B-ESAT-6. No significant protection was found in groups receiving the antigen in DDA or with antigen or BCG lipids alone. Furthermore, nonsignificant levels of protection have been observed with the mycosome preparation alone with a $\log_{10}$ reduction of 0.0 to 0.2 compared to nonimmunized mice (results not shown).

Efficient maintenance of immunological memory by mycosomes. One of the most important features for any new adjuvant system is the maintenance of immunological memory. We assessed the longevity of immunity by measuring responses in the blood and spleen at various time points postimmunization (results not shown). After an initial peak of IFN-γ release at 3 weeks after the final immunization, the levels declined somewhat. However, high levels were still observed as late as 14 months postimmunization (8,953 ± 41 and 1,796 ± 299 pg/ml in the blood and spleen, respectively). The responding cells had a classical effector phenotype characterized by high expression of CD44 and low expression of CD62L (results not shown). Subsequently, the mice were given an aerosol challenge at 2.5 months, 6 months, and 14 months after the first immunization, and the number of CFU in the lungs was monitored. The protection promoted was compared to a standard BCG vaccine. Although BCG gave rise to the highest level of protection at the earliest time point, Ag85B-ESAT-6 emulsified in mycosomes gave rise to high and significant levels of protection at all time points (Fig. 6). Moreover, whereas the protection level of the live BCG vaccine waned over time, the protective efficacy of Ag85B-ESAT-6 administered in mycosomes increased throughout the study period and gave rise to a significantly higher level of protection compared to BCG at the late time point ($P < 0.01$).

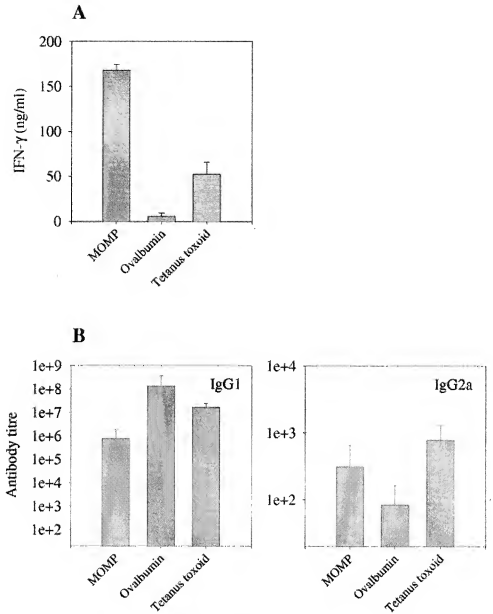

FIG. 5. Induction of immune response using mycosomes as adjuvant for various antigens. (A) Release of IFN-γ from blood lymphocytes isolated from BALB/c mice immunized with 10 μg of MOMP, ovalbumin, or tetanus toxoid in mycosomes. Blood lymphocytes were isolated 5 weeks after the first immunization and restimulated in vitro with the antigen used for immunization (5 μg/ml). (B) Antigen-specific antibody midpoint titers in serum from immunized BALB/c mice measured as IgG1 and IgG2a titers. All results have been compared to naïve mice.

DISCUSSION

Herein, it is demonstrated that a liposomal formulation of mycobacterial lipids is capable of inducing strong humoral as well as cell-mediated immune responses against both mycobacterial and nonmycobacterial antigens. The mycobacterial lipid extract consists of several lipids as demonstrated by TLC and will therefore potentially contain a range of different immunostimulatory molecules. One obvious advantage of using a complex preparation for vaccine delivery is the ability to trigger several components of the proinflammatory cascade, resulting in broader and more sustained biological activity. In this regard, heat-killed *Brucella abortus* has been recognized as a potent inducer of several components of the immune system including antibody secretion and cytotoxic T lymphocyte responses and has shown promise as a delivery system for human immunodeficiency virus antigens (28). Recently, these characteristics were attributed to the interaction of *B. abortus* with several TLRs and the subsequent involvement of the MyD88-signaling pathway (20). Archaeosomes consisting of different lipid moieties from archaea are another example of a complex adjuvant system triggering different immune responses including cytotoxic responses and antibody production (25, 26). Although many of the specific molecules involved in this signaling cascade remain to be identified, it clearly demonstrates the

TABLE 3. Vaccine-induced protection against an aerosol infection with *M. tuberculosis*

Vaccine[a]	Log_{10} resistance ± SEM[b]	
	Lungs	Spleen
Ag85B-ESAT-6	0.01 ± 0.08	0.14 ± 0.08
Ag85B-ESAT-6 + DDA	0.09 ± 0.15	0.28 ± 0.10
Ag85B-ESAT-6 + BCG lipids	0.48 ± 0.08*	0.47 ± 0.16
Ag85B-ESAT-6 + mycosomes	1.19 ± 0.13*	0.97 ± 0.18*
BCG lipids	0.32 ± 0.07	0.29 ± 0.10
BCG	1.10 ± 0.17*	1.30 ± 0.25*

[a] BALB/c mice ($n = 5$) were immunized three times with the indicated experimental vaccines or injected once subcutaneously with BCG.

[b] Number of bacteria from the lungs and spleen 6 weeks after aerosol challenge expressed as log_{10} resistance calculated by subtracting the log_{10} mean number of bacteria in the organs of vaccinated mice from the log_{10} mean number of bacteria in the organs of naïve mice. Values marked with an asterisk are significantly different ($P < 0.01$) compared to naïve controls.

complex interaction between a panel of immunostimulatory molecules from a pathogen and the initiation of a broad and efficient immune response. The identification of receptors through which an innate immune response is triggered is a field in very rapid development (21, 34), and the numbers of identified receptors and, hence, targets for immunomodulation and adjuvant activity are rapidly increasing. An aspect of particular interest in this regard is the role of TLRs in generating and modulating immune responses. Over the last 5 years, this field of research has contributed immensely to our understanding of the mode of action of several of the immunostimulatory molecules that are the backbone of current adjuvant systems. In this regard, both bacterial DNA targeting TLR9 (16) as well as lipid A molecules and synthetically engineered TLR4 agonists have shown promise as vaccine adjuvants (5). The involvement of specific TLRs in relation to the BCG lipid-based adjuvant presented in this paper is the subject of ongoing studies in our laboratory.

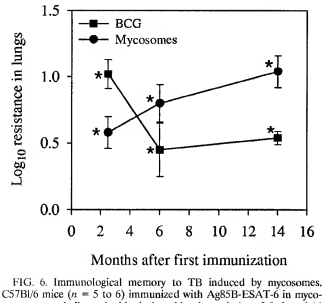

FIG. 6. Immunological memory to TB induced by mycosomes. C57Bl/6 mice ($n = 5$ to 6) immunized with Ag85B-ESAT-6 in mycosomes were challenged with virulent *M. tuberculosis* at 2.5, 6, and 14 months after the first immunization, and the number of CFU ± standard error of the means in lungs was monitored 6 weeks later. Protective effects of the vaccines are expressed as log_{10} resistance calculated by subtracting the log_{10} mean number of bacteria in the lungs of vaccinated mice from the log_{10} mean number in the lungs of unvaccinated control mice. Groups receiving one injection with BCG or non-immunized mice were included as controls. Bacterial numbers significantly different from those seen in control mice are indicated by an asterisk ($P < 0.01$ as assessed by Dunnetts test).

Mycobacteria have long been known to exert a number of immunomodulatory effects and have been used extensively as a source of adjuvant preparations. The best known adjuvant is Freund's complete adjuvant consisting of a paraffin oil emulsion and heat-killed mycobacteria (15); however, live *M. bovis* BCG has also been used as an immunotherapeutic agent (7). Similarly, purified components of mycobacteria have been shown to have immunostimulatory activity. Wax D (a complex of peptidoglycan, arabinogalactan, and mycolic acids) was found to possess strong adjuvant activity (35), and trehalose 6, 6′-dimycolate (or synthetic analogues thereof) has been included in various adjuvant formulations (19, 24, 30). The use of different preparations of mycobacteria in clinical trials involving cancer patients has demonstrated that they also have a use in this setting (4). Hence, Z-100, a lipid arabinomannan extracted from *M. tuberculosis*, was shown to possess antitumor activity (38) and is now clinically used in Japan. Production of immunostimulatory mycobacterial lipid extracts is feasible, whereas the laborious purification schemes of single molecules currently used for therapeutic applications may be too expensive for a future adjuvant formulation for prophylactic use worldwide. Thus, we have used a simple extraction procedure with a starting material already administered extensively worldwide, the BCG vaccine. In addition to accessibility, another advantage of using BCG would be to avoid immunosuppressive molecules associated with clinical isolates of *M. tuberculosis*. In this regard, a phenolic glycolipid identified from the Beijing strain was recently shown to inhibit the release of proinflammatory cytokines (36). At present, optimized BCG extraction protocols and characterization methods for analysis of stability and batch-to-batch variation are being developed in our laboratory to meet the recommendations in the European Medicines Agency guideline on adjuvants in vaccines (14).

In a recent study, liposomes based on phosphatidylinositol mannosides (PIMS) extracted from BCG were investigated as a potential antigen delivery system. The PIMs were able to activate human dendritic cells, and mice immunized with ovalbumin emulsified in PIM liposomes generated ovalbumin-specific antibody and cytotoxic T-cell responses (37). As described in the present paper, we also tried to use the mycobacterial lipids on their own but found a markedly stronger immune response induced when the mycobacterial lipids were administered in combination with cationic liposomes. Indeed, compared to other liposomes, the cationic surfactant DDA stood out as the most efficient vehicle in terms of both antibody production and IFN-γ levels induced. Although DDA has been used as an adjuvant for many decades (for a review, see reference 18) and has even been administered to humans (41), its specific function as an adjuvant is still not fully understood. In the transfection field DDA has proved a very valuable facilitator of gene uptake and is thought to interact via its positive charge with negatively charged cell membranes (43). It may therefore be a similar activity that allows DDA to enhance the

uptake of antigen and the immunomodulatory mycobacterial lipids by antigen-presenting cells.

In the present study one of the most striking abilities of the mycosomes was the very efficient maintenance of immunological memory that even surpassed the memory induced by the live TB vaccine BCG and resulted in efficient protection against TB as late as 14 months postvaccination. In this regard, the formation of a DDA depot ensuring the slow release of antigen has previously been hypothesized as a mechanism by which DDA may function (19). Experiments involving DDA performed by Katz and coworkers showed an antibody response of a longer duration compared to conventional adjuvants such as alum (22), demonstrating the induction of a persistent response with DDA. However, our studies with DDA alone (1) have never resulted in such striking levels of long-term memory as reported in the present study, and, therefore, in addition to the direct effect of DDA, there is undoubtedly an important effect of the mycobacterial lipids. In this regard, a range of mycobacterial lipids, i.e., phthiocerol dimycocerosates and mycolic acids, have been shown to be very resistant to degradation (17), and they may therefore contribute to the long-term effect seen with DDA-BCG lipids in this study. Along these lines, lipid extracts from archaea have also demonstrated high stability and have been found to be resistant to lipase degradation, pH extremes, and temperature variations (12). Indeed, adjuvant preparations based on these lipids (archaeosomes) were found to provide sustained immune responses as assessed by prolonged antibody production (25).

Together with archaeosomes, the present study represents a move toward a less reductionistic approach to adjuvant development. The sustained release of antigen coupled with the continued stimulation of the immune system by the range of stimulatory compounds present in the mycosomes convincingly demonstrates that strong immune responses and long-lived memory are not exclusively provided by live vaccines like BCG but can also be provided by nonreplicating vaccines such as subunit vaccines in an efficient adjuvant.

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